CRYSTALLINE FORM OF FATTY ACID AMIDE HYDROLASE (FAAH)

GOVERNMENT SUPPORT

FIELD OF THE INVENTION

[0002]   The present invention relates generally to structural biology and medicine, especially in the interception of endocannabinoid influence and allied physiological processes. More specifically, the present invention relates to the crystalline form of fatty acid amide hydrolase (FAAH) and the use of these crystals to determine the three-dimensional structure of this protein.

BACKGROUND OF THE INVENTION

[0003]   Fatty acid amide hydrolase (FAAH) is the only characterized mammalian member of the amidase signature (AS) family of serine hydrolases. This family is an ancient and ubiquitous group of enzymes that share an amino acid motif known as the amidase signature. The representatives of this family have a highly diverse array of substrates though they may share a common reaction mechanism. Most AS enzymes described to date hydrolyze amides of small metabolic intermediates, such as acetamide, opines, propionamide, and malonamide. Those AS enzymes described to date also appear to be exclusively soluble proteins.

[0004]   Though a member of the AS family, FAAH possesses unique features which distinguish it from lower homologues. FAAH behaves as an integral membrane protein when isolated from native sources or when expressed recombinantly. The enzyme cannot be separated from membrane fractions with the use of high salt concentrations or alkaline sodium carbonate. It can only be separated from membranes

with the use of detergents. FAAH protein extracted with the aid of non-denaturing detergents retains catalytic activity and hence presumably its native structure.

[0005]   FAAH is present in mammals in various tissues throughout the body, including the brain, liver, duodenum, kidney, and testis. The enzyme is noticeably absent from the heart. In these tissues, the enzyme appears to reside on extensive intracellular membrane systems, likely the smooth endoplasmic reticulum (SER). This conclusion is supported by confocal immunofluorescence data from each of these tissues as well as immunogold electron microscopy of the rat liver. The mechanism by which FAAH is inserted into these membranes and how it is anchored there is currently a subject of active investigation.

[0006]   Unlike its metabolic counterparts in lower organisms, FAAH retains an important role in nervous system function. The substrates of FAAH, the fatty acid amides, have been demonstrated to exert powerful neuromodulatory effects in test animals relating to the physiologies of pain, locomotion, memory, cognition, pyresis, and sleep. The mechanism by which these compounds exert their influence is not yet fully characterized, though a subset of these effects can be abrogated *in vivo* by antagonists of the trimeric G-protein coupled receptor CB1. The fatty acid amides are hydrolyzed by FAAH in an expeditious manner to their pharmacologically inactive acids. Therefore, FAAH acts to terminate the signaling of these molecules and to establish their baseline levels in the cell. As a result, FAAH intersects the physiologies associated with its substrates.

[0007]   *In vivo* demonstrations of the critical role that FAAH plays in mammals has recently been documented with the aid of a genetically engineered mouse model that lacks a functional FAAH gene. These mice possess greatly enhanced sensitivities to the neurological effects of the fatty acid amides when administered by intraperitoneal injection. Further, these mice exhibit an increased tolerance for pain even in the naïve state. Concomitantly, brains from these mice exhibit highly elevated levels of fatty acid amides, suggesting that an absence of FAAH leads to disturbed endogenous fatty acid amide function and results in altered baseline physiology. These observations suggest

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that a pharmacological intervention of the catalytic activity of FAAH may result in similar effects as the present genetic ablation.

[0008]   The catalytic mechanism by which FAAH hydrolyzes its substrates has been investigated and several important findings have resulted. The amino acid residues that are responsible for the active site chemistry have been identified, as has at least one amino acid residue which resides in the substrate binding pocket. One particular finding of interest is the observation that FAAH is able to catalyze the hydrolysis of amides in the presence of equivalent amounts of comparable esters. This feature is contrary to solution chemistry and the action of the unrelated serine hydrolases of the catalytic triad family. How FAAH maintains this preference is currently unknown, though at least one amino acid residue, lysine 142, has been identified which contributes to this effect.

SUMMARY OF THE INVENTION

[0009]   The present invention provides a crystallized mammalian fatty acid amide hydrolase (FAAH) in complex with the active site-directed inhibitor methoxyarachidonyl fluorophosphonate (MAFP). The present invention further provides derivatives of invention FAAH crystals including various heavy metals and methods for the collection of the X-ray diffraction patterns of both native and derivative crystals. The invention also provides methods for analyzing diffraction patterns produced by invention native and derivative crystals by multiple isomorphous replacement (MIR) and single- and multiwavelength anomalous diffraction (SAD/MAD).

[0010]   In one embodiment, there is provided a three-dimensional model for the protein structure of FAAH at the secondary, tertiary, and quaternary levels. Identification of this structural model allows the analysis of FAAH's physicochemical properties and its application to understanding the enzyme's function and mechanism *in vivo*.

[0011]   In another embodiment, there are provided methods for the identification of the active site of FAAH as well as its substrate binding residues. The invention provides the unambiguous localization of the MAFP molecule within the FAAH protein structure, and therefore allows the assignment of amino acid residues that interact with the enzyme's natural substrates as mimicked by the arachidonyl chain of the inhibitor.

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[0012]   In a further embodiment, there are provided methods for characterizing the
physical aspects of the enzyme which allow heterologous agents to enter, interact with, or
otherwise perturb the native structure of the enzyme. The invention describes the
unpredicted presence of a second, alternate route of entry to the active site distinct from
the cellular membrane. Further, the invention provides for the identification of a *src*
homology (SH3) binding domain on an aqueous surface of the enzyme at a quaternary
interface.

[0013]   In a still further embodiment, there are provided methods for identifying,
characterizing, and optimizing agents that interact with the internal channels of FAAH
and therefore stimulate, inhibit, relocalize, stabilize, or destabilize FAAH and/or its
activity. Such an agent might interact with FAAH, for example, at the active site, the
substrate binding pocket, the membrane port, the cytosolic port, the dimerization tunnel,
the membrane-binding domain, the alkyl tunnel, the head group tunnel, and the like. In
another embodiment, there are provided methods for identifying agents that interactb with
the SH3-binding domain and the surface helix-loop-helix. Such an agent may be further
identified, characterized, or optimized by comparison with the MAFP molecule. An
interaction between FAAH and such an agent may be investigated with the aid of manual
or computerized simulation of the interaction between the agent and FAAH. Such a
simulation may then be optimized based on the structure of FAAH and observed
complementarities and incompatibilities between FAAH and the agent under
consideration. The effects of such an agent may then be determined by obtaining the
agent and bringing it in contact with FAAH to measure its effects.

[0014]   In yet another embodiment, there are provided methods for the elucidation
of the three-dimensional structure of a protein or protein complex which is structurally
related to FAAH. In this embodiment, there is provided a method by which a molecule or
complex may be crystallized and X-ray diffraction data obtained from the crystal. The
resulting diffraction data may then be compared with the present known three-
dimensional structure of FAAH, and the structure of the molecule may be determined by
the method of molecular replacement.

[0015]   In a further embodiment, there are provided methods for identifying the
domain of FAAH responsible for the enzyme's association with membranes (membrane-
binding domain, MBD). In this embodiment, there are provided methods wherein the
MBD may be combined with other molecules or molecular complexes to cause a novel
membrane association in the assembly. Additionally, this method provides a means for
removing or mutating the MBD in order to produce FAAH variants that have altered or
abolished membrane-binding characteristics.

[0016]   In yet another embodiment, there are provided methods for altering the
active site of FAAH and its substrate recognition mechanism so as to affect a change in
the enzyme's substrate selectivity. The method provides for the development of FAAH
variants that degrade heterologous compounds. Such FAAH variants may prove useful as
novel chemical catalysts for enantioselective amide hydrolysis.

[0017]   In another embodiment, there are provided methods for screening an agent
for the ability to modulate the activity of FAAH, comprising contacting FAAH with the
agent to form a FAAH-agent complex and measuring the activity level of the FAAH-
agent complex relative to un-complexed FAAH, thereby screening the agent for the
ability to modulate the activity of FAAH.

BRIEF DESCRIPTION OF THE FIGURES

[0018]   Figure 1 illustrates an overview of the three-dimensional structure of fatty
acid amide hydrolase represented in ribbon diagram format. A number of relevant
structural features are highlighted. Parts A and B are related by a 90 degree rotation
about the vertical axis.

[0019]   Figure 2 depicts the active site of FAAH in complex with the arachidonyl
inhibitor MAP.

[0020]   Figure 3 depicts the predicted membrane-binding cap of FAAH.

[0021]   Figure 4 sets forth proposed modular adaptations that convert a soluble
enzyme to an integral membrane enzyme based on differences in the structures of FAAH
and MAE2.

[0022]   Figure 5 depicts exemplary internal channels of FAAH which may be
targeted by a subset of agents which interact with the enzyme.

[0023]   The amino acid sequence of FAAH (SEQ ID NO: 1) is included herein.

DETAILED DESCRIPTION OF THE INVENTION

[0024]   The present invention provides crystalline mammalian fatty acid amide
hydrolase (FAAH) of sufficient quality to produce interpretable X-ray diffraction data.
As used herein, the phrase "fatty acid amide hydrolase (FAAH)" includes any protein
from any mammalian source that displays a catalytic activity to hydrolyze the amide bond
of fatty acid amides and which further contains the amidase signature (AS) sequence.
Such a protein may be derived from primary, recombinant, or synthetic sources. As used
herein, the term "protein" refers to a protein, polypeptide or peptide.

[0025]   FAAH has an amino acid sequence as set forth in SEQ ID NO: 1,
including conservative variations thereof. As used herein, the phrase "conservative
variations" refers to a replacement of an amino acid residue by another, biologically
similar amino acid residue. Examples of conservative variations include the substitution
of a hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or
the substitution of a polar residue for another, such as the substitution of arginine for
lysine, glutamic for aspartic acid, or glutamine for asparagine, and the like.

[0026]   Crystalline FAAH may be produced by combining FAAH protein with a
mother liquor (for example, 100 mM sodium citrate pH 5.0, 100 mM lithium sulfate, 8%
polyethylene glycol (average molecular weight 6000)) and allowing the mixture to vapor
equilibrate with a reservoir of mother liquor for a sufficient time to afford crystals of
FAAH. Suitable crystals are introduced into a collimated X-ray source such that the

crystal may be rotated and a complete diffraction pattern may be recorded. Such a
diffraction pattern may then be analyzed and compared to independent patterns generated
from isomorphous crystals derivatized with heavy metals. Heavy metals contemplated
for use in the practice of the present invention include, osmium, platinum, and the like.

[0027]   Alternately, such derivatized crystals may be exposed to X-rays of suitable
wavelength(s) such that anomalous signals may be obtained for comparison. Such
comparisons may then yield a molecular model of the three-dimensional structure of the
FAAH protein. Such a model may be represented by several means, including computer
records such as protein data bank (pdb) files (see, e.g.,Table II). The invention described
herein includes any representation, including but not limited to, binary records, text
records, graphic representations, and virtual representations, each either physical or
electronic. The present invention also provides for the introduction of an inhibitory
substrate analogue to the protein so that its interactions with the protein may be
determined.

[0028]   FAAH crystals may be expected to take a wide variety of forms, all of
which are included in the present invention. In one embodiment of the FAAH crystal, the
crystal displays primitive monoclinic symmetry P21 and contains sixteen FAAH
molecules per asymmetric unit. The monoclinic embodiment further exhibits
pseudomerohedral twinning as the crystallographic unit cell axes a and c may be
interchanged. In an alternate embodiment, the crystal displays C centered orthorhombic
symmetry C2221 and contains eight molecules per asymmetric unit. In a third
embodiment, the crystal displays primitive hexagonal symmetry P6322 and contains two
molecules per asymmetric unit.

[0029]   The structure further reveals that the FAAH protein does not exist in the
crystal as a monomeric, independent unit, but instead it forms a dimer with simple two-
fold symmetry about an axis parallel to the long axis of the monomer. This arrangement
results in the concerted presentation of helices 15 through 19 from each monomer on the
same face of the assembled dimer. Furthermore, these helices form a protruding, highly
hydrophobic plateau on this face of the dimer. As such, this structural motif provides a

means for interacting with cellular membranes in a specific and stable manner. This face of the enzyme also displays a route of entry to the active site through which substrates or other agents might pass from the membrane. As used herein, "active site" refers to a region of a FAAH that, as a result of its shape and charge potential, interacts with an agent (including, without limitation, a protein, polypeptide, peptide, nucleic acid, including DNA or RNA, molecule, compound, antibiotic or drug).

[0030]   A second route of access to the active site is found on a lateral face of the enzyme near the dimer interface. This second port is marked by histidine 449 and possibly tryptophan 445, each from the polypeptide chain of the dimer mate. A possible mechanism exists by which the enzyme may undergo structural rearrangements so that these ports may be opened and closed in a regulated manner in concert with the progress of the enzymatic reaction or as a means for controlling the enzyme's activity. Such a means of control may result from any number of mechanisms, including, but not limited to, the binding of protein partners or ligands. By means of example but not as a complete explanation, such an effect might be triggered by a representative of the SH3-domain class of proteins. The present structure demonstrates that FAAH displays at its apical surface a consensus polyproline sequence for binding SH3-domain proteins, as well as those of the Homer family. This motif resides on the loop between helices eleven and twelve and includes the amino acid sequence proline-proline-leucine-proline. A potential mechanism exists whereby a regulatory protein such as Homer or an exogenously supplied agent may bind the polyproline sequence on FAAH to introduce a conformational change which might alter the cytoplasmic ports of the enzyme or other structural features to change active site geometry or substrate/product transit.

[0031]   In another embodiment, there are provided methods for identifying agents which interact with the internal channels of FAAH, based on the knowledge of the three-dimensional structure of FAAH, and thereby stimulate, inhibit, relocalize, stabilize, or destabilize FAAH and/or its activity. As used herein, the term "internal channels" refers to several sites within the protein, such as, the active site, the substrate binding pocket, the membrane port, the cytosolic port, the dimerization tunnel, the membrane-binding domain, the alkyl tunnel, the head group tunnel, and the like. The amino acids which

comprise the internal channels of FAAH are set forth in Table 5. In still another
embodiment, there are provided methods for identifying agents which interact with the
SH3-binding domain and the surface helix-loop-helix of FAAH.

[0032]   Such agents may be identified, developed, and characterized based on an
investigation of possible interactions between the agent and the FAAH molecule. This
process might occur by visual inspection of the FAAH structure or, more quantitatively,
with the aid of various computer algorithms designed for such purposes, such as
AUTODOCK, InsightII, and QUANTA. The present invention allows for the virtual
binding of candidate agents *in silico* so that they may be characterized as possible leads
for further study. The effects of such agents may then be tested for activity against
FAAH by bringing the agent into contact with FAAH and determining any effect on the
enzyme's normal function. Such agents identified as candidates by invention methods
will aid in identifying lead compounds for further chemical optimization and/or for
evaluation *in vivo* as potential therapeutics. The invention provides that such optimized
agents may be used in a pharmacological preparation designed to exert physiological
effects when administered to living organisms. Such medically relevant uses for agents
identified or optimized with the use of the present invention include, for example, the
treatment of pain, sleep, addiction, fertility, anxiety, anorexia, fever, cognitive processes,
and the like.

[0033]   A candidate agent can be any type of molecule, including, for example, a
peptide, a peptidomimetic, a polynucleotide, or a small organic molecule, that one wishes
to examine for the ability to act as a therapeutic agent, which is an agent that provides a
therapeutic advantage to a subject receiving it. It will be recognized that a method of the
invention is readily adaptable to a high throughput format and, therefore, the method is
convenient for screening a plurality of test agents either serially or in parallel. The
plurality of test agents can be, for example, a library of test agents produced by a
combinatorial method library of test agents. Methods for preparing a combinatorial
library of molecules that can be tested for therapeutic activity are well known in the art
and include, for example, methods of making a phage display library of peptides, which
can be constrained peptides (see, for example, U.S. Patent No. 5,622,699; U.S. Patent

No. 5,206,347; Scott and Smith, Science 249:386-390, 1992; Markland et al., Gene
109:1319, 1991; each of which is incorporated herein by reference); a peptide library
(U.S. Patent No. 5,264,563, which is incorporated herein by reference); a peptidomimetic
library (Blondelle et al., Trends Anal. Chem. 14:8392, 1995; a nucleic acid library
(O'Connell et al., supra, 1996; Tuerk and Gold, supra, 1990; Gold et al., supra, 1995;
each of which is incorporated herein by reference); an oligosaccharide library (York et
al., Carb. Res., 285:99128, 1996; Liang et al., Science, 274:1520-1522, 1996; Ding et al.,
Adv. Expt. Med. Biol., 376:261-269, 1995; each of which is incorporated herein by
reference); a lipoprotein library (de Kruif et al., FEBS Lett., 399:232-236, 1996, which is
incorporated herein by reference); a glycoprotein or glycolipid library (Karaoglu et al.,
J. Cell Biol., 130:567-577, 1995, which is incorporated herein by reference); or a
chemical library containing, for example, drugs or other pharmaceutical agents
(Gordon et al., J. Med. Chem., 37:1385-1401, 1994; Ecker and Crooke, Bio/Technology,
13:351-360, 1995; each of which is incorporated herein by reference). Accordingly, the
present invention also provides a therapeutic agent identified by such a method.

[0034]   The route of administration of a candidate agent will depend, in part, on
the chemical structure of the candidate agent. Peptides and polynucleotides, for example,
are not particularly useful when administered orally because they can be degraded in the
digestive tract. However, methods for chemically modifying peptides, for example, to
render them less susceptible to degradation by endogenous proteases or more absorbable
through the alimentary tract are well known (see, for example, Blondelle et al., Trends
Anal. Chem. 14:83-92, 1995; Ecker and Crooke, Bio/Technology, 13:351-360, 1995;
each of which is incorporated herein by reference). In addition, a peptide agent can be
prepared using D-amino acids, or can contain one or more domains based on
peptidomimetics, which are organic molecules that mimic the structure of peptide
domain; or based on a peptoid such as a vinylogous peptoid.

[0035]   In another embodiment, there are provided pharmaceutical compositions
including agents identified by invention methods. Invention pharmaceutical compositions
can administered in a variety of ways including, for example, orally or parenterally, such
as intravenously, intramuscularly, subcutaneously, intraorbitally, intracapsularly,

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intraperitoneally, intrarectally, intracisternally or by passive or facilitated absorption through the skin using, for example, a skin patch or transdermal iontophoresis, respectively. Furthermore, the invention pharmaceutical compositions can be administered by injection, incubation, orally or topically, the latter of which can be passive, for example, by direct application of an ointment, or active, for example, using a nasal spray or inhalant, in which case one component of the composition is an appropriate propellant.

[0036]   The total amount of pharmaceutical composition to be administered in practicing a method of the invention can be administered to a subject as a single dose, either as a bolus or by infusion over a relatively short period of time, or can be administered using a fractionated treatment protocol, in which multiple doses are administered over a prolonged period of time. The pharmaceutical composition can be formulated for oral formulation, such as a tablet, or a solution or suspension form; or can comprise an admixture with an organic or inorganic carrier or excipient suitable for enteral or parenteral applications, and can be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers for tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions, or other form suitable for use. The carriers, in addition to those disclosed above, can include glucose, lactose, mannose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea, medium chain length triglycerides, dextrans, and other carriers suitable for use in manufacturing preparations, in solid, semisolid, or liquid form. In addition auxiliary, stabilizing, thickening or coloring agents and perfumes can be used, for example a stabilizing dry agent such as triulose (see, for example, U.S. Pat. No. 5,314,695).

[0037]   In another embodiment, there are provided methods for treating a pathological condition, including administering to a subject in need thereof an invention pharmaceutical composition. In one embodiment, the subject is a mammal. In another embodiment, the subject is human. Pathological conditions that can be effectively treated by invention methods include, for example, anxiety, pain, hunger, sleep, fertility,

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cognition, immunological disorders, fever, tremor, glaucoma, intestinal disorders, and the like.

[0038]   In a still further embodiment, there are provided methods for determining the molecular structure of a molecule or molecular complex whose structure is unknown, comprising:

(a) obtaining crystals of the molecule or molecular complex whose structure is unknown;

(b) generating X-ray diffraction data from the crystallized molecule or molecular complex;

(c) comparing the X-ray diffraction data from the molecule or molecular complex with the three dimensional structure determined from the crystalline form of FAAH; and

(d) using molecular replacement analysis to conform the three dimensional structure determined from the crystalline form of FAAH to the X-ray diffraction data from the crystallized molecule or molecular complex. This method allows for the determination, for example, of a protein which is structurally related to FAAH. As used herein, "structurally related" refers to a protein whose structure can be determined by comparing its X-ray diffraction data to the X-ray diffraction data of FAAH. Such a use of the present invention would include the crystallization of the target protein or protein complex and collection of its X-ray diffraction data. The target data may then be interpreted by means of molecular replacement using the FAAH structure as a template. Such methods obviate the need to collect data from derivatized crystals and greatly simplify the process of solving the structure of the target protein.

[0039]   In a still further embodiment, there are provided methods for engineering proteins with altered physicochemical properties using the structural information obtained from the FAAH crystal. The invention allows for the identification of the membrane-binding domain of the FAAH protein. As such, this domain may be mutated or deleted, in whole or in part, to result in a protein with altered or absent membrane tropism. Further, the identified membrane-binding domain may, in full or in part, be transferred to a heterologous protein so as to affect its membrane tropism. Moreover, the identification

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of the active site and substrate binding pocket of FAAH allows for the targeted generation
of variants with altered substrate specificity and/or altered enzymological properties.
Such variants may prove useful as catalysts of amide hydrolysis unrelated to the fatty acid
amides, and as such may have chemical or industrial applications.

[0040]   The invention will now be described in greater detail by reference to the
following non-limiting example.

EXAMPLE

Crystallization

[0041]   Protein crystals were obtained by recombinantly expressing rat FAAH
with 29 amino acids at the amino-terminus deleted. Though the deleted region is
predicted to participate in membrane binding (8), the truncated FAAH variant (residues
30 - 579) retains the wild-type protein's association with membranes, detergent
requirement for solubilization, and ability to degrade fatty acid amides in mammalian
cells (15). Crystals of FAAH complexed with methoxy arachidonyl fluorophosphonate,
an active site-directed irreversible inhibitor, were obtained in three space groups, $P2_1$ with
sixteen molecules in the assymetric unit, $C222_1$ with eight molecules in the asymmetric
unit, and $P6_322$ with two molecules in the asymmetric unit (Materials and Methods (16)).
The structure of the FAAH-inhibitor complex was first determined to 3.3 Å using a
combination MIRAS / MAD phasing strategy followed by phase extension with multi-
crystal and NCS averaging (Table 1). The resulting electron density maps displayed
density for over 90% of the side chains with no main chain breaks, yielding an
exceptional search model for molecular replacement phasing of twinned monoclinic data.
The final model, built in $P2_1$ at 2.8 Å resolution, was of excellent quality.

Data Collection

[0042]   Table 1 includes a summary of data collection and refinement statistics.
Initial phases were calculated with DmMulti (24), incorporating all MAD and MIRAS

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contributions. Ramachandran distributions of amino acids in the final model were calculated with PROCHECK (24) from a single protein chain after phase refinement under tight NCS restraints. Side chains for which no density was observed were set to zero occupancy during refinement, and no sigma-based rejection of reflections was employed. Numbers in parentheses refer to data in the highest resolution shell. Two residues were in disallowed regions: glutamate 122, which is in the $i+1$ position of a gamma turn, and glutamine 189, which is also in a tight turn immediately followed by a second turn. Table 2 includes the complete data collection and refinement statistics.

Table 1

Data set	TWIN	MAD			
	T1	Ta peak	Ta inflection	Os peak	Remote
Beam line	ALS 5.0.2	SSRL 9-2	SSRL 9-2	SSRL 9-2	SSRL 9-2
Wavelength (Å)	0.97910	1.255	1.2554	1.14025	0.925
Space group	$P2_1$	$C222_1$	$C222_1$	$C222_1$	$C222_1$
Resolution (Å)	50-2.8	65-3.3	65-3.3	60-3.3	48-3.3
Unique reflections	173615	81567	81574	81609	81511
Redundancy	2.1	14.5	14.5	14.5	14.5
Completeness*	76 (79)	99.7 (99.1)	99.7 (99.2)	99.8 (99.5)	99.7 (98.7)
$I/(\sigma I)$*	4.4 (2.7)	8.2 (2.3)	6.9 (1.2)	7.6 (1.7)	8.5 (2.2)
Rsym (%)*	10.4 (26.4)	7.9 (33.3)	9.7 (63.2)	8.5 (45.5)	7.4 (33.4)

Phasing from $Ta_6Cl_{12} \cdot OsCl_3$ MAD data		Refinement against detwinned data set T1	
Resolution (Å)	96-3.3	Resolution (Å)	50-2.8
Number of Os sites	8	Reflections used	173615
Number of Ta sites	0	Test reflections	9297
Os peak isomorphous phasing power (centric/acentric)	0.948/1.43	Number of nonhydrogen atoms	65744
Os peak anomalous phasing power (acentric)	2.18	R_{work} (%)	19.7
Remote isomorphous phasing power (centric/acentric)	0.288/0.471	R_{free} (%)	24.9
Remote anomalous phasing power (acentric)	1.88	Average B value ($Å^2$)	44.829
Ta inflection anomalous phasing power (acentric)	0.871	MAFP average B value ($Å^2$)	64.26
Ta peak (reference) anomalous phasing power (acentric)	1.02	rmsd bond length (Å)	0.079
		rmsd bond angle (°)	2.500
Combined figure of merit before NCS and multi-crystal averaging (3.3 Å) (centric/acentric)	0.47/0.60	Ramachandran distributions (%)	
		Most favored	79.9
		Additionally allowed	17.7
Twinning operator	(l, -h, k)	Generously allowed	2.0
Twinning factor (α)	0.28	Disallowed[†]	0.4

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Table 2

Data set	NATIVES				TWIN	SAD		
	N1	N2	N3	H1	T1	(NH₄)₂PtCl₂	OsCl₃	Ta peak
Beam line	SSRL 9-2	SSRL 11-1	SSRL 11-1	SSRL 9-2	ALS 5.0.2	SSRL 11-1	SSRL 9-2	SSRL 9-2
Wavelength (Å)	1.03317	1.07185	1.07185	0.97946	0.97910	1.07185	1.13973	1.255
Space group	$C222_1$	$C222_1$	$C222_1$	$P6_322$	$P2_1$	$C222_1$	$C222_1$	$C222_1$
Resolution (Å)	60-5.0	70-4.0	64.5-3.8	50-3.5	50-2.8	70-4.0	63-4.0	65-3.3
Unique reflections	19722	46254	53415	18446	173615	45171	46191	81567
Redundancy	4.1	4.8	4.7	20	2.1	9.5	9.5	14.5
Completeness*	83 (83)	99.7 (100)	99.7 (99.7)	99.9 (99.9)	76 (79)	97.7 (98.6)	99.3 (99.0)	99.7 (99.1)
I/σI*	6.0 (2.8)	5.9 (2.6)	9.1 (4.0)	4.6 (1.9)	4.4 (2.7)	4.8 (1.9)	7.0 (3.2)	8.2 (2.3)
Rsym (%)*	10.5 (27.2)	9.7 (26.5)	6.7 (18.6)	12.6 (48.8)	10.4 (26.4)	9.6 (39.4)	9.4 (22.4)	7.9 (33.3)

Phasing from MIRAS data

	N1	N2	N3
Resolution (Å)	48-5.0	48-4.0	48-4.0
Number of Pt sites	13	13	13
Number of Os sites	8	8	8
Pt isomorphous phasing power (centric/acentric)	0.062/0.173	0.107/0.178	0.123/0.181
Pt anomalous phasing power (acentric)	0.583	0.987	0.548
Os isomorphous phasing power (centric/acentric)	0.555/0.692	0.436/0.638	0.408/0.558
Os anomalous phasing power (acentric)	0.767	0.736	0.739

Phasing from Ta₆Cl₁₂ • OsCl₃ MAD data

Resolution (Å)	96-3.3
Number of Os sites	8
Number of Ta sites	0
Os peak isomorphous phasing power (centric/acentric)	0.848/1.43
Os peak anomalous phasing power (acentric)	2.18
Remote isomorphous phasing power (centric/acentric)	0.288/0.471
Remote anomalous phasing power (acentric)	1.88
Ta inflection anomalous phasing power (acentric)	0.871
Ta peak (reference) anomalous phasing power (acentric)	1.02
Combined figure of merit before NCS and multi-crystal averaging (3.3 Å) (centric/acentric)	0.47/0.60

Refinement against detwinned data set T1

Resolution (Å)
Reflections used
Test reflections
Number of nonhydrogen atoms
R_{work} (%)
R_{free} (%)
Average B value (Å²)
MAFP average B value (Å²)
rmsd bond length (Å)
rmsd bond angle (°)
Ramachandran distributions (%)
　Most favored
　Additionally allowed
　Generously allowed
　Disallowed†
Twinning operator
Twinning factor (α)

*Numbers in parentheses refer to data in the highest resolution shell.
†Two residues were in disallowed regions: glutamate 122, which is in the i+1 position of a gamma turn, and glutamine 189, which is also in a tight turn immediately followed by a second turn.

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[0043]   The crystal structure of FAAH reveals a dimeric enzyme (Fig. 1), consistent with chemical crosslinking studies indicating that the enzyme is a dimer in solution (15). The dimer interface buries approximately 1560 $Å^2$ of molecular surface area per monomer (17). The protein core is characterized by a twisted beta sheet consisting of eleven mixed strands which is in turn surrounded by twenty-four alpha helices of varying lengths. The overall fold of the monomer closely resembles that of malonamidase (MAE2) from the nitrogen fixing bacterium *Bradyrhizobium japonicum* (12) and peptide amidase (PAM) from Stenotrophomonas maltophilia (REF: Labahn J, Neumann S, Buldt G, Kula M, Granzin J. J Mol Biol 2002 Oct 4;322(5):1053).

[0044]   However, key structural features make FAAH unique, and these will be discussed below. Further, these variations can be quantified by measuring the root mean squared deviation (rmsd) between FAAH and its bacterial homologues (Tables 3 and 4). If a comparison is drawn between domains of the proteins which obey amino acid sequence alignment, the rmsd difference increases as more atoms are included in the calculation. This is true both in the case of restricting the calculation to an expanding sphere centered on the active site and in the case of expanding the calculation from $C\alpha$s to all atoms. In any case, it is also of note that only a fraction of FAAH can be included in the calculation due to sequence considerations, 42% in the case of PAM and 36% in the case of MAE2 (Table 3). Further, if protein fold topology is taken into consideration, the comparable fractions of FAAH which can be included fall even further, to 22% for PAM and 34% for MAE2 (Table 4).

Table 3. Average rmsd$_{xyz}$ of sequence-based equivalent residue ranges after superposition of catalytic triad Cαs. The number of amino acid residues included in the calculation is indicated.

	5Å	10Å	20Å	100Å
PAM-CHY*				
Cα (222)	1.217	4.443	6.442	8.525
BACKBONE	2.361	4.044	6.452	8.494
ALL ATOMS	2.195	4.486	6.752	8.737
MAE2*				
Cα (191)	0.433	2.191	5.487	9.092
BACKBONE	1.272	2.088	4.634	8.702
ALL ATOMS	1.253	2.363	4.827	8.783

Table 4. Average rmsd$_{xyz}$ of topology-based equivalent residue ranges. The number of amino acid residues included in the calculation is indicated.

	5Å	10Å	20Å	100Å
PAM-CHY*				
Cα (116)	0.415	1.136	5.473	5.449
BACKBONE	0.553	1.126	5.350	5.488
ALL ATOMS	0.504	1.126	5.147	5.290
MAE2*				
Cα (178)	0.561	1.044	1.146	1.384
BACKBONE	0.661	1.028	1.221	1.429
ALL ATOMS	0.606	1.134	1.341	1.664

*Insertions/deletions and non-identical amino acid side chains are not included in the calculation. PAM-CHY is the complex of peptide amidase and the inhibitor chymostatin.

[0045]   The active site of FAAH was identified based on the location of core catalytic residues defined previously by mutagenesis (*10, 18*) and from the density of the inhibitor adduct methoxy arachidonyl phosphonate (MAP) (Fig. 2). The catalytic nucleophile, serine 241, is covalently bonded to the phosphorous of the MAP molecule,

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and the neighboring density can be modeled to accommodate the arachidonyl chain in an energetically favorable conformation (Fig. 2A). As observed for MAE2, the serine nucleophile in FAAH forms part of an unusual serine-serine-lysine catalytic triad with serine 217 and lysine 142 (Fig. 2B), and the relative orientation of the catalytic residues is nearly superimposable between the structures of FAAH and MAE2 (rmsd=0.27 Å). In contrast, the substrate-binding pockets of FAAH and MAE2 differ significantly. The tunnel leading from the surface of FAAH and containing the buried arachidonyl chain is lined with a preponderance of aromatic and aliphatic amino acids (Fig. 2B), many of which arise from sequence insertions in FAAH relative to MAE2. These residues include isoleucine 491, identified by UV cross-linking and mutagenesis studies to participate in substrate recognition (*14*). In contrast, the active site of MAE2 is lined with mostly hydrophilic amino acids that accommodate its soluble substrate, malonamide (*12*).

[0046]   Amino acids 410 to 438, another sequence insertion in FAAH relative to MAE2, form a helix-turn-helix motif that interrupts the AS fold. The two helices, α18 and α19, cap the active site and present several hydrophobic residues that likely compose FAAH's membrane binding face (Figs. 1, 3A). The *N*-terminus of the intact enzyme, predicted by sequence analysis to form an additional membrane-binding helix (amino acids 7-29), would be properly positioned to reinforce the membrane interactions of the α18 and α19 membrane cap (Fig. 1B).

[0047]   A potential substrate entryway is adjacent to α18 and α19, and the arachidonyl chain of MAP contacts phenylalanine 432 of α18, which may indicate direct access between the FAAH active site and the hydrophobic membrane bilayer. The putative substrate entry is amphipathic in nature with hydrophobic residues covering three sides of the rim and charged residues arginine 486 and aspartate 403 completing the remaining side (Fig. 2A, 3B). This arrangement of residues may accommodate the admission and movement of polar fatty acid amide head groups towards the active site. Overall, the intimate relationship between the membrane binding surface and active site of FAAH is similar to that of squalene cyclase (*19*) and prostaglandin H_2 synthase (*20*) which also act upon lipid soluble substrates and have hydrophobic caps surrounding their respective active site entrances. In all three enzymes, the hydrophobic cap is surrounded by basic amino acids (Fig. 3B) that may interact with negatively charged phospholipids.

[0048]   A second major tunnel emerges from the active site at an approximately 80
degree angle from the arachidonyl-filled cavity. This tunnel bifurcates to create both a
solvent-exposed cytosolic port and a route blocked by tryptophan 445 (Fig. 2A), a residue
that forms a lock-and-key intersubunit contact. Thus, the FAAH active site appears to
simultaneously access both the aqueous environment of the cytoplasm and the lipid
milieu of the bilayer. This architecture may provide an exit route to the cytosol for the
polar amine substituents liberated from the fatty acid amide substrates and could also
provide entry for a water molecule required for deacylation of the FAAH-fatty acyl
intermediate (18).

[0049]   Overall, a comparison of FAAH's structure to that of the soluble AS
enzyme MAE2 reveals how a single member of a large clade of proteins has adapted to
perform a specialized cellular function through the addition of discrete folding modules
and without gross changes in catalytic mechanism or fold architecture (Fig. 4). For
example, although FAAH and MAE2 dimerize about roughly the same face, they have
different relative monomer orientations. These distinct quarternary orientations produce
an antiparallel and parallel alignment of the MAE2 and FAAH monomer active site
entrances, respectively. The parallel orientation of FAAH monomers has important
biological implications, as it should permit both subunits to function concurrently by
recruiting substrates from the same membrane. Furthermore, this parallel alignment
places another key structural module, the $\alpha18$-$\alpha19$ hydrophobic cap, on the same face of
the dimer, thereby enhancing membrane binding (Fig. 4). Additional sequence insertions
in the FAAH protein account for other specialized features of the enzyme's structure,
including its cytoplasmic channel and apolar substrate binding pocket (Fig. 4).

[0050]   The intimate relationship between the active site of FAAH and cell
membranes revealed by the enzyme's structure raises the possibility that fatty acid amides
need not be transported through aqueous cellular compartments in order to proceed from
site of action to site of degradation. As a consequence, proper endocannabinoid tone may
rely on both the expression levels of FAAH and its localization relative to CB receptor
systems in vivo (21). Finally, the structure of FAAH may facilitate the design of
inhibitors that could have therapeutic benefit for a variety of nervous system disorders
(22), including not only agents that bind to the enzyme acyl chain pocket, but also

compounds that target the active site via the cytoplasmic channel. This latter class of inhibitors might display exceptional selectivity for FAAH relative to the more than one hundred lipid hydrolases present in the human proteome.

[0051]   Figure 1 depicts the structure of the integral membrane protein fatty acid amide hydrolase (FAAH). The enzyme is a homodimer assembled from 63 kDa subunits. The inhibitor adduct methoxy arachidonyl phosphonate (MAP) is depicted in the active site with van der Waal surfaces rendered in yellow (carbon), gray (phosphorous), and red (oxygen). (A) Front view of the enzyme, highlighting the central twisted beta sheet that forms the core of the structure. (B) Side view produced by a 90° rotation from view A. The amino (N) and carboxyl (C) termini of the green subunit are labeled. Proline 129 is indicated. A genetic polymorphism conferring substitution of threonine at this position has been implicated in drug abuse and renders the enzyme more vulnerable to proteolytic attack (25). All figures were produced with Molscript (26), GRASP (23), and Raster3D (27). The membrane model was generated by Molecular Dynamics simulation of a palmitoyloleoylphosphatidylethanolamine (POPE) bilayer (28).

[0052]   Figure 2 depicts the active site of FAAH in complex with the arachidonyl inhibitor MAP. (A) The FAAH dimer is shown with the protein surface rendered in gray (hydrophilic) and green (hydrophobic) for one subunit and pale yellow for its mate. A portion of the protein surface has been removed to highlight the continuous internal channel leading from the membrane binding face at arginine 486 (blue) and aspartate 403 (red) to the active site and on to the cytosolic port. Electron density corresponding to the arachidonyl inhibitor is shown in violet and lies in the hydrophobic (green) substrate-binding pocket. Tryptophan 445 from the dimer mate is rendered in van der Waals surface and colored cyan to demonstrate the effective plugging of this potential port. (B) Aromatic and aliphatic residues in the substrate binding pocket surrounding the arachidonyl chain and their interactions are indicated. Also shown is the unusual serine-serine-lysine catalytic triad of FAAH (ser241-ser217-ser241), with the serine 241 nucleophile covalently bonded to the phosphorus atom of the MAP inhibitor.

[0053]   Figure 3 depicts the predicted membrane-binding cap of FAAH. (A) The hydrophobic helices α18 and α19 that comprise the putative membrane-binding cap are

shown in green. The primary sequence of this domain (404-433) is indicated using amino acid one letter code (*29*) except for hydrophilic amino acids, which are indicated by *x*. Five of these seven hydrophilic residues are arginine or lysine; the remaining two are serines. (B) The molecular surface of FAAH viewed from the membrane face; the observed structure demonstrates the presence of a hydrophobic cap (*top, green*), surrounded primarily by positive electrostatic potential (*bottom; blue, basic; red, acidic*). The entrance to the active site from the membrane face is indicated, as are arginine 486 and aspartate 403, which form one side of this access port.

[0054]   Figure 4 sets forth proposed modular adaptations that convert a soluble enzyme to an integral membrane enzyme based on differences in the structures of FAAH and MAE2. The soluble enzyme, if oligomeric, reorganizes so that all active sites can concurrently access the bilayer for maximal efficiency. The addition of discrete structural elements, represented here in red, confer the new oligomerization domain (tryptophan 445 and residues 299-314; *lower left panel*) as well as a membrane binding face (*lower middle left panel*) and cytoplasmic access channel (*lower middle right panel*). The final monotopic integral membrane enzyme must also undergo mutation of key substrate binding residues in the active site to effectively recruit its hydrophobic targets from the lipid bilayer (*lower right panel*).

Table 5. Residues expected to line the interior channels of FAAH. The coordinates of the Cαs in the present structure are provided as a guide only.

ATOM		0	CA	LYS	A	142	12.558	-31.943	29.679	0.00	0.00
A	C										
ATOM		0	CA	SER	A	190	17.871	-20.245	32.364	0.00	0.00
A	C										
ATOM		0	CA	MET	A	191	17.760	-22.751	29.499	0.00	0.00
A	C										
ATOM		0	CA	LEU	A	192	19.766	-20.186	27.640	0.00	0.00
A	C										
ATOM		0	CA	SER	A	193	22.793	-22.387	27.483	0.00	0.00
A	C										
ATOM		0	CA	PHE	A	194	24.110	-25.405	25.603	0.00	0.00
A	C										
ATOM		0	CA	SER	A	217	19.643	-27.223	27.046	0.00	0.00
A	C										
ATOM		0	CA	THR	A	236	12.999	-27.716	22.998	0.00	0.00
A	C										
ATOM		0	CA	ILE	A	238	15.579	-22.197	19.986	0.00	0.00
A	C										

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ATOM        0  CA   GLY A 239      19.319 -22.114  19.282  0.00  0.00
A      C
ATOM        0  CA   GLY A 240      20.185 -25.826  19.177  0.00  0.00
A      C
ATOM        0  CA   SER A 241      19.006 -27.246  22.499  0.00  0.00
A      C
ATOM        0  CA   PHE A 244      23.393 -30.169  20.863  0.00  0.00
A      C
ATOM        0  CA   THR A 257       9.128 -26.186  12.131  0.00  0.00
A      C
ATOM        0  CA   ASN A 259       4.442 -24.354  14.717  0.00  0.00
A      C
ATOM        0  CA   LEU A 261       3.547 -28.979  17.435  0.00  0.00
A      C
ATOM        0  CA   SER A 262       1.827 -27.120  20.226  0.00  0.00
A      C
ATOM        0  CA   LYS A 263       3.541 -25.099  22.924  0.00  0.00
A      C
ATOM        0  CA   SER A 264       0.514 -24.586  24.939  0.00  0.00
A      C
ATOM        0  CA   LEU A 266       5.685 -24.877  27.877  0.00  0.00
A      C
ATOM        0  CA   GLY A 268       9.936 -20.496  29.403  0.00  0.00
A      C
ATOM        0  CA   CYS A 269      11.633 -17.639  31.102  0.00  0.00
A      C
ATOM        0  CA   VAL A 270      10.747 -14.992  28.633  0.00  0.00
A      C
ATOM        0  CA   TYR A 271       7.552 -14.969  26.504  0.00  0.00
A      C
ATOM        0  CA   GLY A 272       6.363 -13.140  23.340  0.00  0.00
A      C
ATOM        0  CA   GLN A 273       9.614 -13.020  21.432  0.00  0.00
A      C
ATOM        0  CA   THR A 274       8.660 -14.154  17.873  0.00  0.00
A      C
ATOM        0  CA   ALA A 275      11.611 -13.454  15.736  0.00  0.00
A      C
ATOM        0  CA   VAL A 276      13.533 -16.679  15.922  0.00  0.00
A      C
ATOM        0  CA   GLN A 277      11.336 -19.629  16.496  0.00  0.00
A      C
ATOM        0  CA   LEU A 278      10.970 -21.950  19.360  0.00  0.00
A      C
ATOM        0  CA   ASP A 306      -4.793 -26.348  20.414  0.00  0.00
A      C
ATOM        0  CA   THR A 308      -3.495 -22.832  16.561  0.00  0.00
A      C
ATOM        0  CA   VAL A 309      -1.332 -25.679  15.400  0.00  0.00
A      C
ATOM        0  CA   TYR A 335      32.027 -18.555  10.993  0.00  0.00
A      C
ATOM        0  CA   LEU A 372      23.776 -14.742  12.937  0.00  0.00
A      C
ATOM        0  CA   GLU A 373      24.892 -11.299  14.242  0.00  0.00
A      C
ATOM        0  CA   SER A 376      19.587 -14.121  15.269  0.00  0.00
A      C
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ATOM	0	CA	ALA	A	377	21.364	-12.481	18.109	0.00	0.00
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ATOM	0	CA	LEU	A	380	17.049	-12.894	20.827	0.00	0.00
A	C									
ATOM	0	CA	PHE	A	381	18.126	-9.780	22.708	0.00	0.00
A	C									
ATOM	0	CA	PHE	A	388	15.472	-12.482	30.704	0.00	0.00
A	C									
ATOM	0	CA	LEU	A	401	26.491	-19.448	32.271	0.00	0.00
A	C									
ATOM	0	CA	GLY	A	402	29.874	-17.666	32.101	0.00	0.00
A	C									
ATOM	0	CA	ASP	A	403	29.566	-13.889	31.936	0.00	0.00
A	C									
ATOM	0	CA	LEU	A	404	26.384	-13.628	29.944	0.00	0.00
A	C									
ATOM	0	CA	ILE	A	407	27.407	-8.419	30.439	0.00	0.00
A	C									
ATOM	0	CA	ARG	A	428	32.279	-10.875	16.349	0.00	0.00
A	C									
ATOM	0	CA	LEU	A	429	32.511	-9.785	19.879	0.00	0.00
A	C									
ATOM	0	CA	PHE	A	432	27.625	-9.601	20.031	0.00	0.00
A	C									
ATOM	0	CA	TRP	A	445	9.219	-4.401	13.406	0.00	0.00
A	C									
ATOM	0	CA	GLN	A	448	10.948	-9.045	11.948	0.00	0.00
A	C									
ATOM	0	CA	HIS	A	449	10.701	-7.903	8.430	0.00	0.00
A	C									
ATOM	0	CA	ILE	A	451	13.771	-11.849	8.960	0.00	0.00
A	C									
ATOM	0	CA	GLU	A	452	10.849	-12.835	6.872	0.00	0.00
A	C									
ATOM	0	CA	MET	A	453	12.858	-11.529	3.862	0.00	0.00
A	C									
ATOM	0	CA	ARG	A	455	13.826	-16.561	4.859	0.00	0.00
A	C									
ATOM	0	CA	GLN	A	456	12.307	-15.855	1.397	0.00	0.00
A	C									
ATOM	0	CA	PRO	A	484	29.797	-23.817	26.313	0.00	0.00
A	C									
ATOM	0	CA	GLY	A	485	29.335	-20.718	28.271	0.00	0.00
A	C									
ATOM	0	CA	ARG	A	486	32.477	-19.218	26.838	0.00	0.00
A	C									
ATOM	0	CA	THR	A	488	28.751	-18.591	22.179	0.00	0.00
A	C									
ATOM	0	CA	GLY	A	489	29.978	-18.362	18.590	0.00	0.00
A	C									
ATOM	0	CA	ILE	A	491	25.976	-22.214	18.037	0.00	0.00
A	C									
ATOM	0	CA	SER	A	492	26.470	-20.646	14.610	0.00	0.00
A	C									
ATOM	0	CA	VAL	A	495	20.892	-21.229	15.354	0.00	0.00
A	C									
ATOM	0	CA	CYS	A	499	16.329	-18.125	12.495	0.00	0.00
A	C									

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ATOM        0  CA  LEU A 500        15.055 -19.509   9.162  0.00  0.00
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ATOM        0  CA  ASP A 501        12.509 -21.783  10.900  0.00  0.00
A       C
ATOM        0  CA  ILE A 530        38.543 -15.553  22.466  0.00  0.00
A       C
ATOM        0  CA  TRP A 531        35.687 -17.843  21.343  0.00  0.00
A       C
ATOM        0  CA  ILE A 534        38.136 -21.645  24.267  0.00  0.00
A       C
ATOM        0  CA  TRP A 556         6.679 -28.127   7.026  0.00  0.00
A       C
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SEQ. ID. NO 1:

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  1 mvlsevwttl sgvsgvclac sllsaavvlr wtgrqkarga atrarqkqra sletmdkavq
 61 rfrlqnpdld sealltlpll qlvqklqsge lspeavffty lgkawevnkg tncvtsyltd
121 cetqlsqapr qgllygvpvs lkecfsykgh dstlglslne gmpsesdcvv vqvlklqgav
181 pfvhtnvpqs mlsfdcsnpl fgqtmnpwks skspggssgg egaligsggs plglgtdigg
241 sirfpsafcg icglkptgnr lsksglkgcv ygqtavqlsl gpmardvesl alclkallce
301 hlftldptvp plpfreevyr ssrplrvgyy etdnytmpsp amrralietk qrleaaghtl
361 ipflpnnipy alevlsaggl fsdggrsflq nfkgdfvdpc lgdlililrl pswfkrllsl
421 llkplfprla aflnsmrprs aeklwklqhe iemyrqsvia qwkamnldvl ltpmlgpald
481 lntpgratga isytvlyncl dfpagvvpvt tvtaeddaqm elykgyfgdi wdiilkkamk
541 nsvglpvavq cvalpwqeel clrfmreveq lmtpqkqps
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29. A, alanine; F, phenylalanine; L, leucine; I, isoleucine; P, proline; W, tryptophan.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.